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mglur6b:EGFP transgenic zebrafish suggest novel functions of metabotropic glutamate signaling in retina and other brain regions

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Abstract

Metabotropic glutamate receptors (mGluRs) are mainly known for regulating excitability of neurons. However, mGluR6 at the photoreceptor-ON bipolar cell synapse mediates sign inversion through glutamatergic inhibition. Although this is currently the only confirmed function of mGluR6, other functions have been suggested. Here, we present *Tg(mglur6b:EGFP)zh1*, a new transgenic zebrafish line recapitulating endogenous expression of one of the two *mglur6* paralogues in zebrafish. Investigating transgene as well as endogenous *mglur6b* expression within the zebrafish retina indicates that EGFP and *mglur6b* mRNA are not only expressed in bipolar cells, but also in a subset of ganglion and amacrine cells. The amacrine cells labeled in *Tg(mglur6b:EGFP)zh1* constitute a novel cholinergic, non-GABAergic, non-starburst amacrine cell type described for the first time in teleost fishes. Apart from the retina, we find transgene expression in subsets of periventricular neurons of the hypothalamus, Purkinje cells of the cerebellum, various cell types of the optic tectum and mitral/ruffed cells of the olfactory bulb. These findings suggest novel functions of mGluR6 besides sign inversion at ON bipolar cell dendrites opening up the possibility that inhibitory glutamatergic signaling may be more prevalent than currently thought.

Introduction

Glutamate is the major excitatory neurotransmitter in the central nervous system, including the retina. Both ionotropic and metabotropic receptors exert wide-spread functions of glutamate in neuronal signaling. Ionotropic glutamate receptors (iGluRs) are nonselective cation channels that directly generate an excitatory signal upon glutamate binding. In contrast, metabotropic glutamate receptors (mGluRs) modulate ion channels indirectly through several intracellular signaling cascades. mGluRs can be divided into three types based on pharmacology, signaling pathways activated and similarity in amino acid sequence (Conn and Pin, 1997). Type I mGluRs are generally postsynaptic and stimulate phospholipase C through Gq/11 protein signaling. Conversely, type II and type III mGluRs predominantly lie presynaptically where they activate Gi/o proteins, thereby inhibiting cAMP production and consequently reducing protein kinase A activity.

In addition, type III mGluRs are known as auto- and heteroreceptors regulating neurotransmitter release, a function that mostly relies on cAMP independent regulation of ion channels. In particular, type III mGluRs inhibit Ca^{2+} channels and activate K^{+} channels (Mercier and Lodge, 2014). Moreover, they have been proposed to regulate neurotransmitter release by directly acting on the exocytotic machinery (Anwyl, 1999). There are four members of type III mGluRs in mammals: mGluR4, mGluR6, mGluR7 and mGluR8. Among them, mGluR6 stands out due to its atypical postsynaptic localization. In the mammalian retina, it mGluR6 was reported to be exclusively localized to dendritic tips of ON bipolar cells (Nomura et al., 1994). ON bipolar cells, in contrast to sign-preserving OFF bipolar cells, reverse the sign of the photoreceptor signal due to mGluR6 signaling: In darkness, photoreceptors are depolarized and continuously release glutamate, which is sensed by mGluR6 at the postsynapse. Upon glutamate binding, mGluR6 interacts with the effector G protein Goα (Nawy, 1999; Dhingra et al., 2000), which mediates closure of the constitutively

active cation channel TRPM1 (Koike et al., 2010; Morgans et al., 2009), leading to hyperpolarization of the ON bipolar cell. Because of its crucial role in retinal signaling, mutations in human *MGLUR6* cause congenital stationary night blindness, accompanied by typical loss of the ON response in the electroretinogram (Dryja et al., 2005). Consistently, absence of mGluR6 results in impaired ON signaling in animal models (Huang et al., 2012; Masu et al., 1995; Zeitz et al., 2005). Besides mGluR6 playing a direct role in synaptic transmission, it is also necessary for postsynaptic localization of proteins involved in the ON signaling cascade (Cao et al., 2009; Morgans et al., 2007) and the Ca^{2+} channel subunit Cacna1s (Tummala et al., 2014).

While the role of mGluR6 in ON bipolar cells has been well described, reports suggesting additional functions of mGluR6 are scarce. In adult rodents, *mglur6* transcripts were never found in healthy retinal ganglion cells, but expression was activated in juvenile ganglion cells and adult ganglion cells after optic nerve injury in rats (Tehrani et al., 2000). In transgenic mice that express enhanced green fluorescent protein (*EGFP)* under the *mglur6* promoter (Dhingra et al., 2008), EGFP was detected in amacrine cells and retinal ganglion cells during early embryonic development (Morgan et al., 2006) as well as in certain brain areas, such as accessory olfactory bulb. Human retinal ganglion cells also express *MGLUR6*, (Klooster et al., 2011) and *mglur6* transcripts were even identified outside the mouse nervous system (Vardi et al., 2011).

Work in zebrafish has clearly shown that the two *mglur6* paralogs present in the zebrafish genome, *mglur6a* and *mglur6b*, are not only expressed in bipolar cells, but also in the proximal inner nuclear layer, retinal ganglion cells and various additional brain regions (Haug et al., 2013; Huang et al., 2012).

Here, we present a new transgenic zebrafish line, in which an *mglur6b* upstream regulatory region drives *EGFP* expression. Using this line, we could identify a novel cholinergic amacrine cell type in the retina and demonstrate a potential role of mGluR6 signaling in neurons within a number of brain regions, including projection neurons of the olfactory bulb.

Materials and Methods

Fish maintenance and breeding

Zebrafish were kept at 26°C under a 14/10-hour light/dark cycle as previously described (Mullins et al., 1994). Embryos were raised at 28°C in E3 medium and staged according to development in days post fertilization (dpf) (Kimmel et al., 1995). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local authorities (Veterinäramt Zürich TV4206).

Creation of *Tg(mglur6b:EGFP)* construct

A 4.8 kb fragment upstream of *mglur6b*, ending at position -16 relative to the *mglur6b* translational start site, was PCR amplified from BAC clone DKEY-13A21 (Source BioScience) using a proof-reading Polymerase (Phusion High-Fidelity DNA Polymerase, FINNZYMES). PCR primers with attB4 (forward primer) and attB1 (reverse primer) sites added to 5' ends were used to allow Gateway cloning of the amplified fragment (forward primer: GGGGACAACTTTGTATAGAAAAGTTGACAGGCTACGGATATTTCAGTTC; reverse primer: GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCGGCTCGTTGTTCTTCTAC).

Recombination reactions were performed according to the Gateway® Three-Fragment Vector Construction Kit (Invitrogen, Life Technologies). Briefly, a BP recombination reaction was performed to integrate the amplified fragment into pDONRP4-P1R (Invitrogen), resulting in a 5' entry clone suitable for Multisite Gateway cloning. Restriction and partial sequencing confirmed identity and correct insertion of the *mglur6b* upstream element. To create the *Tg(mglur6b:EGFP)* expression construct (Fig. 1A), we performed a multisite LR reaction of our 5' entry clone, pME-EGFP (middle entry clone), p3E-polyA (3' entry clone) and pDestTol2CG2 (destination vector; all vectors from Tol2 kit, (Kwan et al., 2007). Again, correct assembly of the resulting *Tg(mglur6b:EGFP)* vector was checked and confirmed by restriction and partial sequencing.

Transposase mRNA synthesis

pCS2FA-transposase plasmid (Kwan et al., 2007) was linearized with NotI. Capped RNA was transcribed using mMessage mMachine SP6 kit (Ambion), phenol:chloroform extracted and precipitated using isopropanol according to the manufacturer's instructions.

Generation of *Tg(mglur6b:EGFP)zh1* zebrafish

25 pg of *Tg(mglur6b:EGFP)* and 25 pg transposase mRNA were co-injected into one-cell stage zebrafish embryos resulting in EGFP expression in the heart (transgenesis marker expression) and eyes (reporter gene expression). Larvae with EGFP expression in the eyes detectable under a fluorescent Stereomicroscope (Olympus MVX10) were raised to adulthood and outcrossed to wild type (wt) fish. Progeny of 7 *mglur6b:egfp* injected fish were analyzed for transgene expression. Offspring of 3 injected fish had detectable EGFP expression in the eye, with variable rates of transgenesis marker and eye expression between these different founders. F1 larvae showing EGFP expression in the eye were raised to adulthood. Three F1 fish were again outcrossed to wt, all crosses resulting in F2 offspring

with clear transgene expression in the eye. Two of these fish, one with strong and one with moderate EGFP expression, were used as founders for *mglur6b:EGFP* transgenic lines. Upon further outcrossing to wt, offspring of the F1 fish with only moderate EGFP expression had lost EGFP expression in the eyes in the F3 generation. However, EGFP expressed in the eyes of F3 offspring of the other F1 fish was clearly visible under a stereomicroscope (Fig. 2A). This transgenic line was therefore used for all further experiments presented here and is termed *Tg(mglur6b:EGFP)zh1*.

Live observation and live imaging

After gastrulation but before 1dpf, embryos were treated with 3 μM PTU (1-phenyl-2-thiourea, Sigma-Aldrich) to suppress pigmentation. For observation, larvae were anesthetized with Tricaine (MS-222, Sigma-Aldrich). For live imaging, anesthesized larvae were mounted in 1% low melting temperature agarose (Nu Sieve GTG agarose, Lonza) in E3 medium and covered with E3 medium containing Tricaine. Larvae were observed and imaged using a fluorescent stereomicroscope (Olympus MVX10) equipped with a color camera (ColorViewIII, Soft Imaging System, Olympus).

Whole-mount immunostaining

PTU-treated larvae were fixed in 4% paraformaldehyde for 1 hour at room temperature, dehydrated in an ascending Methanol series (25%, 50%, 75% in PBS and 100%), rehydrated, permeabilized in Acetone (7 minutes at -20°C) followed by two washes in ddH_2O and blocked for 30 minutes at room temperature in PBDT (1% bovine serum albumin, 1% DMSO, 0.1% Triton-X in PBS) supplemented with 10% goat serum. Larvae were incubated in chicken anti-GFP antibody (Table 1) diluted in PBDT supplemented with 2% goat serum overnight at 4°C. Secondary antibody (Alexa conjugated goat anti-chicken, 1:500; Molecular Probes, Life Technologies) was diluted in PBDT and applied for 2 hours at room

temperature. Larvae were incubated in Glycerol for at least 1 hour and mounted in Glycerol containing 1.5% low melting temperature agarose (Nu Sieve GTG agarose, Lonza) for imaging.

Immunostaining on sections

Adult zebrafish were euthanized using Tricaine. All tissue was fixed in 4% paraformaldehyde at 4°C. For adult brain sections, the head was cut and fixed overnight. For adult retinal sections, eyes were excised and fixed for 1 hour. Larvae were fixed overnight. Section immunohistochemistry was carried out as described previously (Rinner et al., 2005), except that tissue was embedded in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Fisher Scientific) and cryosectioned at 16 µm thickness. When a primary antibody raised in goat was applied, blocking solution without goat serum was used. For primary antibodies and concentrations, see Table 1. Goat, donkey or rabbit anti-chicken, anti-rabbit, anti-mouse or anti-goat IgG conjugated to Alexa 488 or 568 fluorophores (Molecular Probes, Life Technologies), were used at dilutions 1:500 - 1:1000. BODIPY TR Methyl Ester (Molecular Probes, Life Technologies; 1:300) was applied for 20 min after secondary antibodies and nuclei were conterstained with DAPI. Sections were coverslipped with Mowiol mounting medium containing DABCO.

Antibody characterization

Please see Table 1 for a list of all primary antibodies used.

The PKCβ1 antibody recognized only bands of around 70 kD on Western blots of cell lysates and does not recognize other human PKC isoforms (manufacturer's datasheet). It stained a pattern of cellular morphology and distribution in the zebrafish retina consistent with ON

bipolar cell labeling and previous reports (Biehlmaier et al., 2003; Yazulla and Studholme, 2001).

The ChAT antibody recognized only a band of around 70 kD on Western blots of mouse brain lysate (manufacturer's information at www.merckmillipore.com), and stained a pattern of cellular morphology and distribution consistent with amacrine cell labeling, identical to previous reports from the teleost retina (Yazulla and Studholme, 2001; Maurer et al., 2010).

The GAD65/67 antibody recognized two bands of expected sizes on Western blots of rat brain lysates (Braden et al., 2010), and stained a pattern of cellular morphology and location in the zebrafish retina consistent with previously used markers for GABAergic amacrine cells in zebrafish (Yazulla and Studholme, 2001).

The TH antibody recognized only a band of around 60 kD on Western blots of cell lysates (manufacturer's datasheet), and stained a pattern of cellular morphology and location in the zebrafish retina identical to previous reports using another TH antibody (Maurer et al., 2010). Double labeling with this second TH antibody (Millipore, AB152, RRID:AB_390204) resulted in complete overlap of staining in the retina (not shown).

RNA *in situ* hybridization

A DIG-labeled RNA probe complementary to nucleotides -10 to 1675 of *mglur6b* mRNA was prepared and chromogenic *in situ* hybridization was performed as previously described (Huang et al., 2012) on adult brain cross sections.

Combined fluorescent RNA *in situ* hybridization and immunohistochemistry

Fluorescent RNA *in situ* hybridization was performed as previously described (Huang et al., 2012). For signal development, Alexa Tyramide 488 (TSA Kit#12, Molecular Probes, Life Technologies) was used. Subsequent to staining for *mglur6b* mRNA, immunohistochemistry

using chicken anti-GFP antibody was carried out as described above with the alteration that primary antibody incubation was performed for 1h at RT. As secondary antibody, goat anti-chicken IgG conjugated to Alexa 568 was used. Complete bleaching of transgenically expressed EGFP during the course of *in situ* hybridization was checked and confirmed in a control experiment using Alexa Tyramide 568 instead of Alexa Tyramide 488 and omitting the antibody staining.

Image acquisition and processing

Images of whole mount larvae and overview images of adult brain sections were acquired on a Leica HCS LSI confocal microscope equipped with a 5x zoom objective (Leica Microsystems). Larval sections, adult retinal sections and details of adult brains were imaged on the same Microscope equipped with a 40x oil objective and on a Leica SP5 confocal microscope (Leica Microsystems). Images were adjusted for brightness and contrast using ImageJ (RRID:SCR_003070). Figures were composed with Adobe Photoshop CS6 (RRID:SCR_014199).

Results

Retinal transgene expression verifies specificity of *Tg(mglur6b:EGFP)* construct

In order to generate a *Tg(mglur6b:EGFP)* transgenic construct, we cloned a 4.8 kb long upstream fragment of *mglur6b* including the first intron (Fig. 1A) into a Tol2 transgenesis vector (Kwan et al., 2007) that contains a *cmlc2:EGFP* transgenic heart marker for better selection (Fig.1B) (for details see Materials and Methods).

Around 20% of injected embryos that were positive for the transgenic heart marker showed detectable EGFP expression in the eye (data not shown). Immunohistochemistry revealed EGFP expression in cells of the inner nuclear layer (INL) and the ganglion cell layer (GCL) (Fig.1C), matching the endogenous *mglur6b* expression pattern (Huang et al., 2012). We therefore conclude that the 4.8 kb regulatory region of *mglur6b* contains elements sufficient and necessary for tissue specific *mglur6b* expression. Consequently, we raised a stable transgenic line from injected founders (see Methods), termed *Tg(mglur6b:EGFP)zh1*.

EGFP expression pattern in the developing central nervous system suggests yet unidentified *mglur6b* expression domains

EGFP expression in the eyes of *Tg(mglur6b:EGFP)zh1* larvae can be observed from 3 dpf onwards (Fig. 2A,B). Histological sections reveal EGFP positive cells in the INL and GCL (Fig.2C), just as in *Tg(mglur6b:EGFP)* injected fish (Fig.1C). Based on morphology and location of EGFP positive cell bodies in the INL, we conclude that a subset of bipolar and amacrine cells are labeled. In addition, a subpopulation of ganglion cells expresses EGFP, evident by partial labeling of the optic nerve (Fig. 2C). We cannot exclude however that at least some of the labeled cells in the GCL are displaced amacrine cells.

Additional EGFP expression in a number of brain regions (Fig.2B,D,E) becomes evident by confocal microscopy. For identification of labelled brain regions and their nomenclature, we generally referred to the zebrafish brain atlas of larval (Mueller and Wullimann, 2005) and adult (Wullimann et al., 1996) zebrafish. In the optic tectum, not only terminals of retinal ganglion cells are EGFP positive, but also cell bodies within the optic tectum (Fig.2B,D) are weakly labeled. Furthermore, EGFP positive cells are present in the cerebellum (Fig.2B), dorsal thalamus (Fig.2D), migrated area of eminentia thalami (Fig.2E) and the hypothalamus (Fig. 2D). In the telencephalon, both pallium and subpallium contain labelled cells (Fig2E,F).

However, by far the strongest signal can be seen in a subset of olfactory bulb neurons and projectons to the habenula (Fig.2G) (Miyasaka et al., 2009), indicating specific transgene expression in olfactory bulb projection neurons. These findings suggest the discovery of yet unidentified *mglur6b* expression domains.

EGFP expression in the brain is maintained up to adulthood

We next examined transgene expression in the adult brain. Similar to larvae, EGFP expression occurs in optic tectum, cerebellum, thalamic region, hypothalamus, pallium, subpallium and most prominently in the olfactory bulb. Thus, expression in *Tg(mglur6b:EGFP)zh1* appears to remain largely unchanged after 5 dpf.

For our following analysis, we focus on selected brain regions which show strong or cell type specific expression. Our approach is particularly suited for cell-type specific *mglur6b* expression analysis, since transgenically expressed EGFP labels entire neurons including their processes, yielding information about their shape and connectivity.

Olfactory bulb projection neurons express *mglur6b*

In *Tg(mglur6b:EGFP)zh1*, the olfactory bulb shows strong EGFP expression in its outer region, whereas the central area is largely devoid of signal, with the exception of few weakly labeled cells, likely being olfactory bulb interneurons (Fig.3A,B). The outer region of the zebrafish olfactory bulb is mainly populated by mitral and ruffed cells, constituting the olfactory bulb projections neurons. (Byrd and Brunjes, 1995; Fuller and Byrd, 2005; Fuller et al., 2006; Rink and Wullimann, 2004). The typical ruffed cell morphology, characterized by elaborate membrane protrusions (ruffs) at the initial segment of the axon, can easily be observed in EGFP expressing cells (Fig.3B3,4). Ruffs can be found on many, but not all labelled cells in the olfactory bulb outer regions. Therefore, very likely mitral cells also

express EGFP (Fig. 3B5,6). Since mitral and ruffed cells are the projection neurons of the olfactory bulb, the lateral olfactory tract (Fig.3A) is also labeled, confirming labeling of olfactory bulb output neurons. Fluorescent RNA *in situ* hybridization using an *mglur6b* antisense probe in combination with antibody staining for EGFP shows that *mglur6b* is indeed strongly expressed in ruffed and probably also mitral cells (Fig.4A).This suggests that mGlur6b may play a role in olfactory processing, at least on the level of olfactory bulb output neurons.

***Mglur6b* is expressed in distinct cell types of the optic tectum**

The optic tectum of teleost fishes is a major projection area for retinal ganglion cells, integrating and processing sensory information. The laminar structure of the optic tectum harbors a deep cell body layer (periventricular grey zone, PGZ) and a superficial neuropil layer containing axons and dendrites from cells of PGZ (Nevin et al., 2010). The majority of EGFP expressing cell bodies within the optic tectum is located in the PGZ (Fig.3E). A few EGFP positive cell bodies can additionally be found within the neuropil layer. In some of them, heavily branched projections to the outermost layer of the optic tectum can be observed (Fig.3E), reminiscent of pyramidal cells known from the optic tectum of other teleost fishes (Vanegas and Ito, 1983; Meek and Schellart, 1978). We also found EGFP expression in cell bodies residing distally to the major projection lamina of retinal ganglion cells (stratum fibrosum et griseum superficiale), representing superficial interneurons (Nevin et al., 2010). Since the optic tectum contains a population of cholinergic cells (Mueller et al., 2004), we checked whether EGFP expressing cells are also positive for acetylcholinesterase (ChAT). However, both in larvae and adults, EGFP and ChAT label non-overlapping subpopulations of tectal neurons (Fig.5). RNA *in situ* hybridization shows that not only *EGFP*, but also *mglur6b* is expressed in the adult optic tectum (Fig.4C). These findings suggest a more widespread role of mGluR6 in the visual pathway than previously described, extending

mGluR6 function from retinal signaling to signaling in the major visual processing center of the brain.

Labeling of ON and OFF bipolar cell subtypes in *Tg(mglur6b:EGPF)zh1*

EGFP expression in the adult retina is found in the same layers as in larvae, consistent with the previously published adult retinal expression pattern of *mglur6b* (Huang et al., 2012). *Tg(mglur6b:EGFP)zh*1 shows labeling of bipolar cells and amacrine or displaced ganglion cells within the INL, and ganglion cells and potentially displaced amacrine cells in the GCL (Fig.6).

In the central INL of the retina, numerous cells with typical bipolar cell morphology are labeled. Double-labeling with PKCβ1, a marker for ON bipolar cells, shows substantial overlap of EGFP and PKCβ1 positive cells (Fig.7A-D). However, not all ON bipolar cells are labeled in *Tg(mglur6b:EGPF)zh1*. Roughly one sixth of PKCβ1 positive cells are negative for EGFP. The vast majority of these cells show the typical morphology of the s6L bipolar cell with its big axon terminal in the proximal IPL (Connaughton et al., 2004). Conversely, more than 1/3 of EGFP positive cells do not co-label with PKCβ1 and are located distally to all PKCβ1 positive terminals (Fig.7A-C), putting into question the expectedly exclusive transgene expression in bipolar cells of the ON type. Indeed, close examination of the IPL shows that there are bipolar cell boutons labeled in the distal half of the IPL (Fig.7E-H), generally considered the OFF sublamina. All known mixed ON/OFF bipolar cell types (Connaughton et al., 2004) have axon terminals in sublaminae devoid of EGFP positive bipolar cell terminals. Taken together, these findings indicate labeling of OFF bipolar cells in *Tg(mglur6b:EGPF)zh1*.

Tg(mglur6b:EGPF)zh1 reveals a novel amacrine cell type

To further elucidate the identity of labeled cells in the proximal INL of the retina, we performed immunohistochemistry for known markers of amacrine cells. EGFP positive cells in the proximal INL are all positive for ChAT, a marker for cholinergic neurons (Fig.8A-C). ChAT is prominently expressed in starburst amacrine cells across diverse species (Masland and Tauchi, 1986; Vaney, 1990; Yazulla and Studholme, 2001). Absence of overlapping EGFP and ChAT immunoreactivity (Fig.8A-C) in the GCL argues however against transgene expression in starburst amacrine cells, which typically show a mirror-like arrangement in INL and GCL. Moreover, unlike starburst amacrine cells, the EGFP expressing cells in *Tg(mglur6b:EGFP)zh1* are not GABAergic, as revealed by staining for GAD65/67 (Fig.8D-F). Due to the low number of EGFP positive cells in the proximal INL we suspected that labeled cell might be dopaminergic. However, tyrosine hydroxylase immunoreactivity, marking dopaminergic neurons, does not overlap with EGFP staining (Fig.8G-I) suggesting that these EGFP positive cells represent an amacrine cell type described for the first time in teleost fishes.

Discussion

In this study, we generated a new transgenic zebrafish line, *Tg(mglur6b:EGFP)zh1*, in order to identify brain regions and neuronal cell types using mGluR6b to sense glutamate. We employed live imaging and immunohistochemistry to visualize EGFP expressing cells and RNA *in situ* hybridization to detect *mglur6b* transcripts. mGluR6 is so far only known for its role in sign inversion at the ON bipolar cell synapse, and expression is generally considered to be restricted to ON bipolar cells. Therefore, it seems surprising that transgene as well as *mglur6b* mRNA expression (this study and Haug et al., 2013).

are found in a number of other retinal cell types and specific brain regions, including olfactory bulb and optic tectum. Our findings open the exciting possibility that mGluR6 mediated sign inversion plays a role at other synapses besides the photoreceptor-ON bipolar cell synapse, which would imply that glutamatergic inhibition in the vertebrate central nervous system is more common than currently appreciated.

EGFP expression recapitulates *mglur6b* mRNA expression

Within the *Tg(mglur6b:EGFP)zh1* retina, subpopulations of bipolar, amacrine and ganglion cells are EGFP positive. In addition, EGFP is expressed in cell bodies within optic tectum, hypothalamus, cerebellum, thalamus, olfactory bulb and in some cells of the remaining telencephalon. These regions were labeled in larval as well as adult zebrafish, and transgene expression is largely consistent with previously published *mglur6b* expression patterns in larval zebrafish (Haug et al., 2013; Huang et al., 2012). However, *mglur6b* expression has not been reported from optic tectum, thalamus and cerebellum, and expression has not yet been analyzed in adult brain. Here, we were able to confirm *mglur6b* expression in the adult olfactory bulb (Fig, 4A), hypothalamus (Fig.4B) and optic tectum (Fig.4C) by RNA *in situ* hybridization. The fact that transgene and mglur6b mRNA expression in the optic tectum are weak compared to other expression domains likely accounts for not having detected it previously in larvae.

Interestingly the habenula has been shown to be weakly *mglur6b* mRNA positive (Haug et al., 2013), but the transgene is absent in habenular cell bodies. As expression in the olfactory bulb is very strong, and trafficking of mRNAs coding for synaptic proteins is known to occur (Zivraj et al., 2010; Taylor et al., 2009), this discrepancy might be explained by the fact that *mglur6b* mRNA could be located in axons of olfactory bulb neurons rather than habenular

cell bodies. Double labeling of *mglur6b* mRNA and EGFP directly demonstrated transgene and *mglur6b* mRNA expression in the same olfactory bulb neurons.

Multiple roles of mGluR6b in visual signal processing

EGFP and *mglur6b* mRNA are expressed in multiple cell types contributing to visual signal processing: Within the retina, where visual information is perceived, filtered and further processed, transgene expression is evident in subtypes of bipolar, amacrine and ganglion cells. Within the optic tectum, constituting the major brain area for integrating visual and other sensory information in teleost fishes, several cell types displaying distinct locations and morphologies are labeled. Transgene expression in tectal superficial interneurons is intriguing, since these cells have been proven necessary for size filtering (Del Bene et al., 2010), and it would be interesting to see whether mGluR6b contributes to this function.

In line with reported expression and function of mGluR6 across vertebrates, double labeling with PKCβ confirmed transgene expression in ON bipolar cells. The existence of PKCβ / EGFP negative cells demonstrates that transgene expression is restricted to a subpopulation of ON bipolar cells. PKCβ positive / EGFP negative bipolar cells constitute one specific bipolar cell type, the Bon-s6L cell (Connaughton et al., 2004) , which can easily be identified by strong PKCβ labeling, a big cell body and a big axon terminal in the proximal INL. This cell type is known to receive predominant input from rods (Li et al., 2012). Interestingly, it has been previously suggested that the rod pathway relies more on the receptor encoded by the *mglur6a* paralogue, while *mglur6b* is more important for the cone ON pathway (Haug et al., 2013). Our finding supports this hypothesis.

Unexpectedly, we also detected EGPF positive / PKCβ negative cell bodies and EGFP positive bipolar cell terminals within the inner plexiform OFF sublayer, two findings suggesting transgene expression in OFF bipolar cells. Due to the high degree of overlap

between *mglur6b* and EGFP expression in *Tg(mglur6b:EGFP)zh1*, we consider this a very likely possibility. Alternatively, EGFP positive axon terminals within the upper half of the inner plexiform layer may actually belong to ON bipolar cells, as they are located in proximity to the ON sublayer. This would however imply that the subdivision of the inner plexiform layer generally agreed on in zebrafish (Connaughton et al., 2004) is not completely accurate. Electrophysiological examination would be needed to resolve whether the transgene is indeed expressed in OFF bipolar cells. If *mglur6b* is expressed in OFF bipolar cells as suggested, we expect it to have a role other than sign inversion at the photoreceptor synapse, implying that mGluR6 may not be entirely specialized on glutamatergic inhibition.

Interestingly, transgene expression also reinforces the surprising finding of *mglur6b* expression in retinal ganglion cells (Huang et al., 2012). Despite a strong tenet of mGluR6 expression being restricted to bipolar cells, there is literature suggesting mGluR6 expression in retinal ganglion cells even outside teleost fishes (Tehrani et al., 2000; Morgan et al., 2006; Klooster et al., 2011). In murine ganglion cells, mGluR6 was only detected during development and after injury (Tehrani et al., 2000), pointing to a role in development or plasticity. The situation might be different in zebrafish, as expression in retinal ganglion cells is maintained up to adulthood, arguing rather for a role in synaptic modulation than in development.

mGluR6b signaling in olfactory bulb and other brain areas

EGFP and *mglur6b* mRNA are strongly expressed in projection neurons of the olfactory bulb, raising the exciting possibility that mGluR6b has a hyperpolarizing role in mitral/ruffed cell inhibitory coupling, similar to its established function in the photoreceptor-ON bipolar cell synapse. Interestingly, transgene expression has been previously reported in accessory

olfactory bulb mitral cells of mGluR6 transgenic mice (Vardi et al., 2011). Although this study did not directly address endogenous mGluR6 expression, together with our findings it suggests evolutionary conserved mGluR6 signaling in the olfactory bulb. However, it will require physiological studies to see whether mitral/ruffed cells receive inhibitory glutamatergic input similar to retinal ON bipolar cells.

Within the cerebellum, EGFP is expressed in the Purkinje cell layer, characterized by its location between granule cell and molecular layer. Since endogenous mRNA expression has not been reported from the cerebellum, it remains to be determined whether mGluR6b plays a role in cerebellar signaling. Both the EGFP transgene and *mglur6b* mRNA are also expressed in the hypothalamus (Haug et al., 2013). To our knowledge, the hypothalamus is the only brain region apart from the retina that clearly has been reported to express *MGLUR6* in mammals (Ghosh et al., 1997). This conserved expression points to an important role of mGluR6 in hypothalamic function.

Discovery of a novel amacrine cell type by *Tg(mglur6b:EGFP)zh1*

In the zebrafish retina, 28 morphologically distinct amacrine cell types have been described so far (Jusuf and Harris, 2009). Like other vertebrates, zebrafish possess cholinergic amacrine cells (Yazulla and Studholme, 2001). Cholinergic amacrine cells have been generally termed starburst amacrine cells due to their morphology, and they have been shown to be essential for direction selectivity of retinal ganglion cells. GABA, which is also released by starburst amacrine cells, is important for conveying this direction selectivity (Taylor and Smith, 2012; Caldwell et al., 1978). To our surprise, we found transgene expression in cholinergic, non GABA-ergic amacrine cells that also do not display typical mirror-like arrangement across the inner plexiform layer. Therefore, *Tg(mglur6b:EGFP)zh1* labels an amacrine cell type not previously described in teleost fishes. While starburst amacrine cells are often considered the

only cholinergic retinal cells, there is a number of reports demonstrating cholinergic cells of non-starburst type in mammalian and non-mammalian retinae (Guiloff and Kolb, 1992; Millar et al., 1985; Schmidt et al., 1985; Conley et al., 1986; Sandmann et al., 1997). Most similar to our finding, cholinergic, non-GABAergic amacrine cells lacking typical starburst cell arrangement were found in the proximal INL of the ground squirrel retina (Cuenca et al., 2003). Since the role of cholinergic synapses in retinal signaling is still poorly understood, the function of this cell type is currently unknown.

Conclusion

We have generated a new transgenic zebrafish line, *Tg(mglur6b:EGFP)zh1*, well recapitulating endogenous *mglur6b* expression. Expression of the transgene as well as *mglur6b* in cells besides the previously described ON bipolar cells strongly argues for additional function of this receptor besides its role at the photoreceptor-ON bipolar cell synapse. In particular, our data extend mGluR6b mediated glutamate signaling to multiple levels of visual signal processing including the optic tectum, and to other brain areas, most prominently the olfactory bulb.

It is very likely that such additional functions also exist outside of teleost fishes, as indicated by a number of reports (Tehrani et al., 2000; Dhingra et al., 2008; Klooster et al., 2011; Vardi et al., 2011; Ghosh et al., 1997). Which specific role mGluR6b plays in these other neuronal cell types remains an open question. Two mutually non-exclusive possibilities seem most likely: First, mGluR6b might have a neuromodulatory role, similar to reported function of other group III mGluRs. Second, mGluR6b might mediate synaptic transmission with glutamate acting as inhibitory neurotransmitter, similar to its role in ON bipolar cells. Although glutamate is generally considered an excitatory neurotransmitter, there are reports showing cases of glutamatergic inhibition in both invertebrates (Chalasani et al., 2007; Liu

and Wilson, 2013) and vertebrates (Lee and Sherman, 2009; Cox and Sherman, 1999; Sekizawa et al., 2009), and mGluRs of group II and III have been shown to be responsible for these effects in vertebrates.

To shed more light on novel mGluR6 functions, it will be important to establish subcellular localization of mGluR6b protein, since postsynaptic localization would be in favor of a role in direct synaptic transmission. Further, physiological studies of EGFP expressing cells in *Tg(mglur6b:EGFP)zh1* will give us more insight into their function and the function of mGluR6b. This would be particularly interesting in combination with pharmacological inhibition of mGluRs or genetic knockout of *mglur6b*, which, with the establishment of CRISPR/Cas9 system (Hwang et al., 2013), is now readily possible in zebrafish.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. SMGK: study concept and design, establishing transgenic line, data acquisition and analysis, drafting, writing and revision of the manuscript; RW: establishing transgenic line, revision of the manuscript; MG: study design, revision of the manuscript; SCFN: study supervision, concept and design, drafting and revision of the manuscript.

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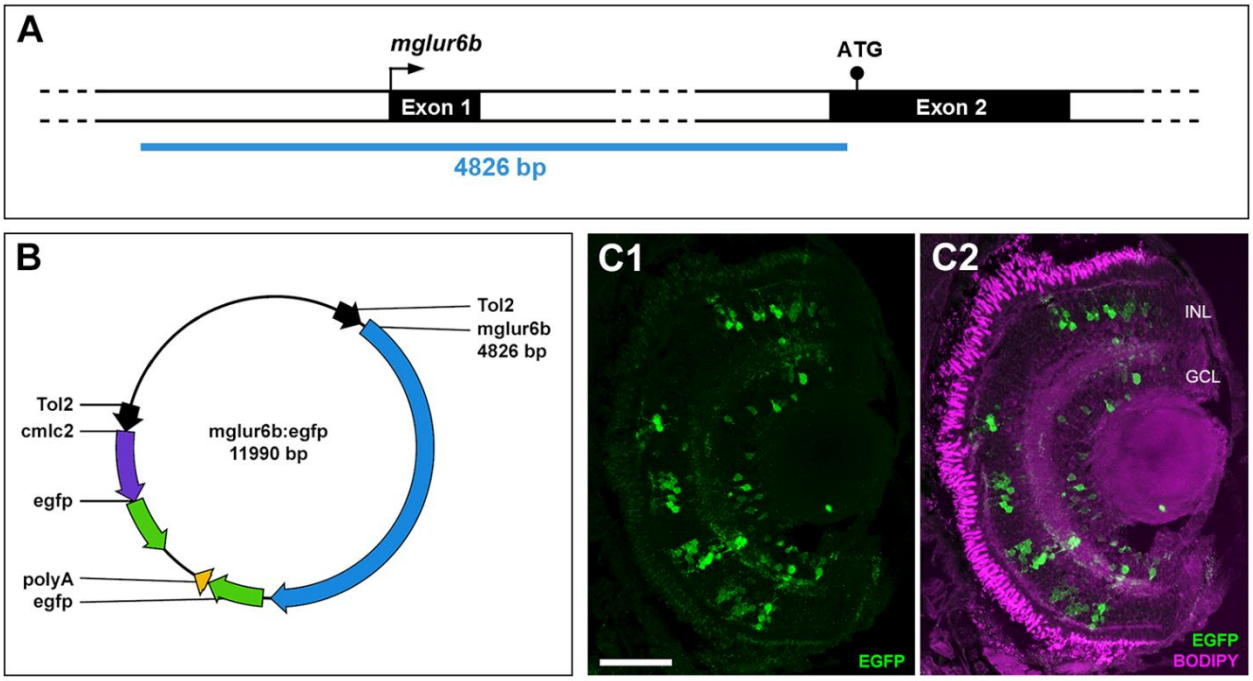

Figure 1. *Tg(mglur6b:EGFP)* vector construct and transient EGFP expression. A 4826 bp long region upstream of the zebrafish *mglur6b* translational start site **(A)** was isolated by PCR and integrated together with EGFP coding sequence and polyA signal in a transgenesis vector containing Tol2 sites and *cmlc2:EGFP* as transgenesis marker by Multisite Gateway cloning, resulting in *Tg(mglur6b:EGFP)* **(B)**. Injection of *Tg(mglur6b:EGFP)* together with transposase mRNA led to EGFP expression in the retinal inner nuclear layer (INL) and ganglion cell layer (GCL) in 5-day-old zebrafish larvae as revealed by immunohistochemistry on larval cross sections **(C)**. Scale bar corresponds to 50 µm. GCL, ganglion cell layer; INL, inner nuclear layer.

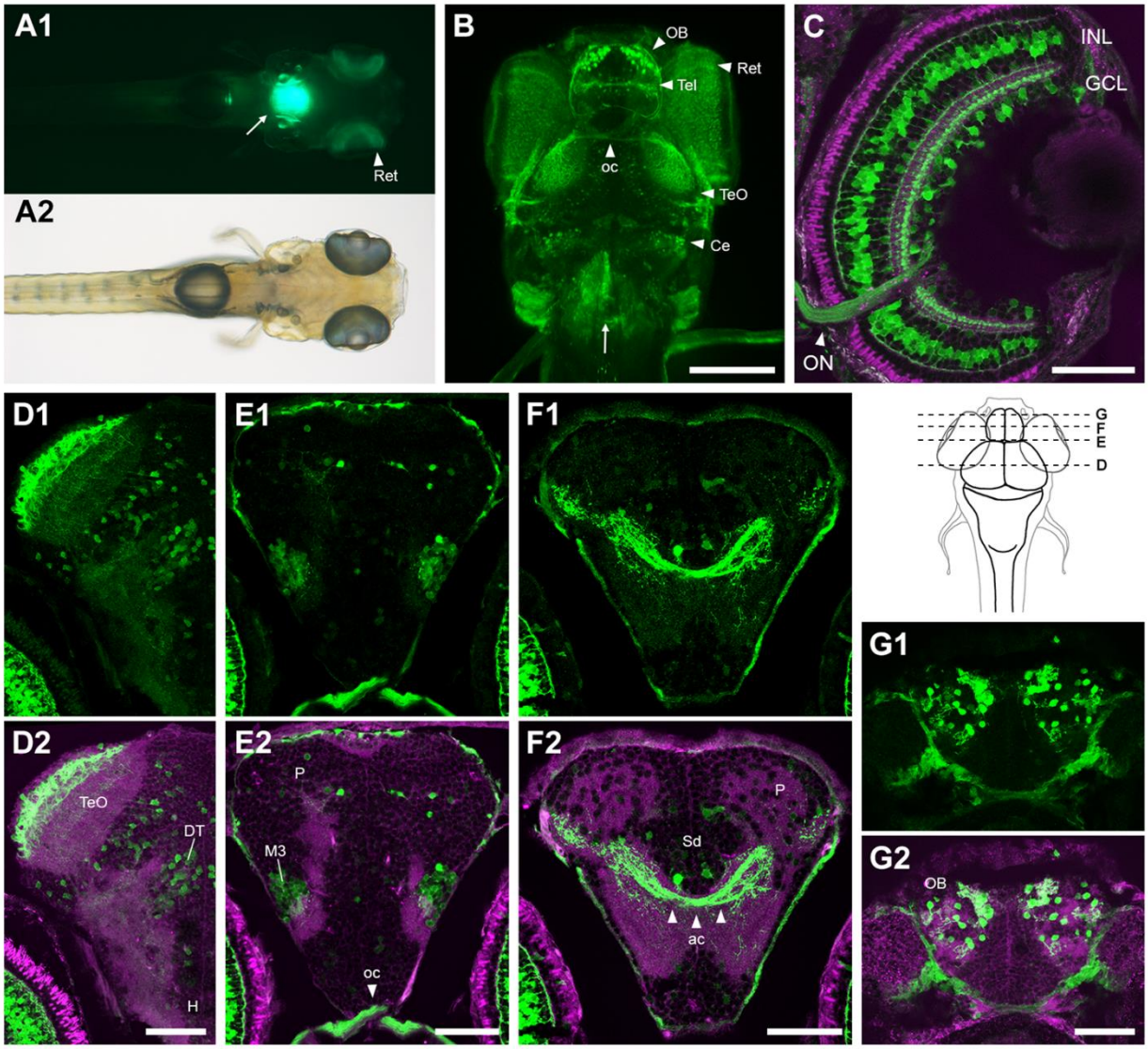

Figure 2. EGFP expression in 5-day-old *Tg(mglur6b:EGFP)zh1* zebrafish. Live imaging **(A),** whole-mount immunohistochemistry **(B)** and immunohistochemistry on cross sections of 5-day-old zebrafish **(C-G).** Wide-field live observation **(A)** shows EGFP expression in the retina. Green fluorescence of the heart **(A,B**, arrows**)** is due to expression of the *cmlc2:EGFP* transgenesis marker contained in the transgenesis vector. Whole-mount immunohistochemistry followed by confocal microscopy **(B)** reveals additional EGFP expression in cell bodies of the olfactory bulb (OB), a small number of other telencephalic neurons (Tel), the optic tectum (TeO) and the cerebellum (Ce). Note that also optic nerves (ON) emerging from retinal ganglion cells **(C)**, crossing in the optic chiasm (oc) **(B,E)** and projecting to the TeO **(B,D)** are labeled. Within the retina, EGFP is expressed in the INL and GCL. Cross sections through the brain imaged with confocal microscopy reveal EGFP in cell bodies of the TeO **(D)**, dorsal thalamus (DT) **(D)**, hypothalamus **(D)**, pallium (P) **(E,F)**, migrated area of eminentia thalami (M3) **(E)**, dorsal division of subpallium (Sd) **(F)** and OB **(G)**. Green, EGFP; Magenta, BODIPY. Scale bars correspond to 200 µm **(B)** and 50 µm **(C-E)**. ac, anterior commissure; Ce, cerebellum ; DT, dorsal thalamus; GCL, ganglion cell layer; EmT, eminentia thalami; H, hypothalamus; INL, inner nuclear layer; M3, migrated area of eminentia thalami; OB, olfactory bulb; oc, optic chiasm; ON, optic nerve; P, pallium; Ret, retina; Sd, dorsal division of subpallium; Tel, telecephalon; TeO, optic tectum.

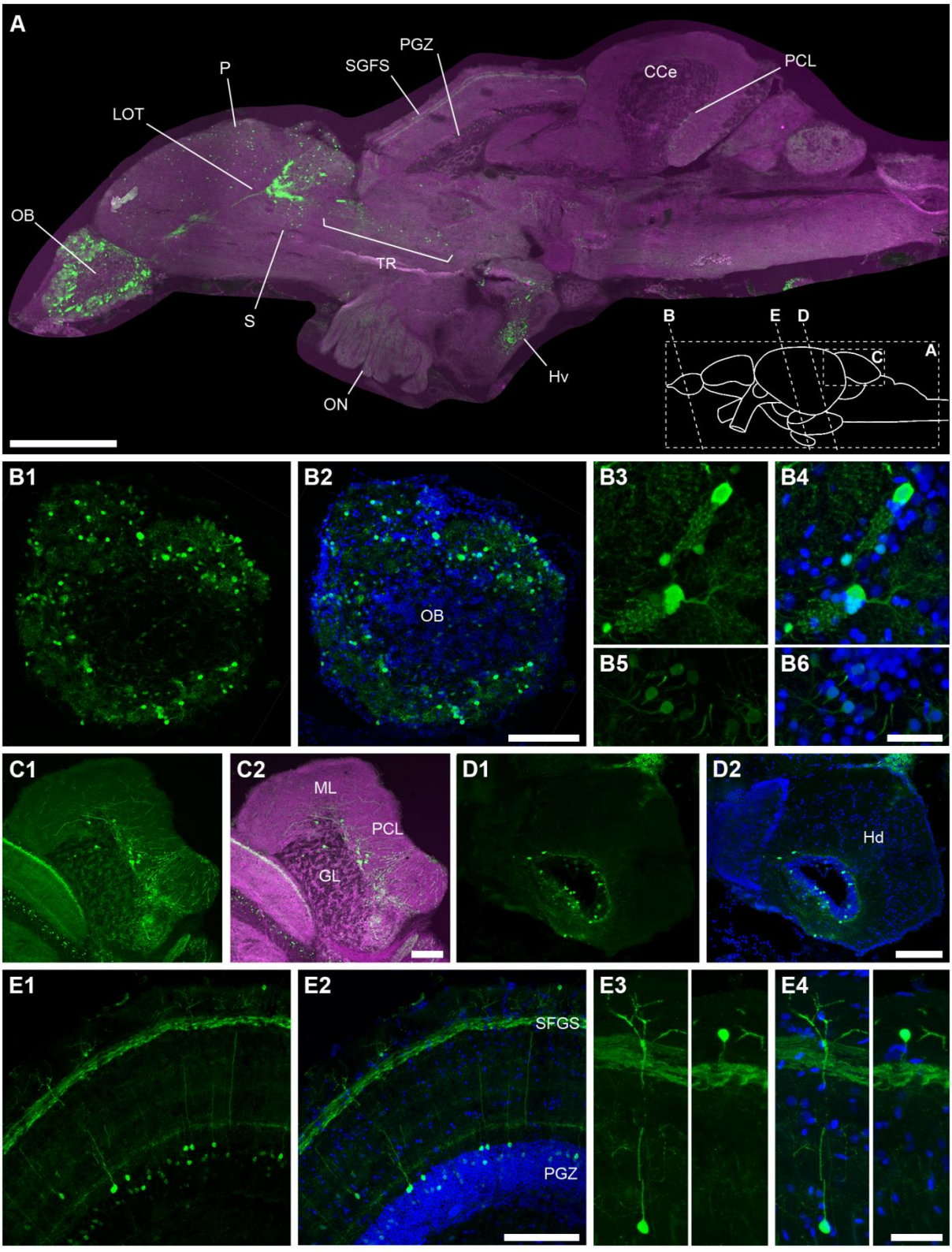

Figure 3. EGFP expression in adult *Tg(mglur6b:EGFP)zh1* zebrafish brain. Confocal images of sagittal **(A,C)** and cross sections **(B,D,E)** of adult zebrafish brain stained with EGFP antibody. The OB is strongly labeled in its outer region **(A,B)**, and higher magnification reveals strong labeling of mitral/ruffed cells with their typical morphology, whereas some other OB neurons (granule cells or interneurons) express EGFP weakly in comparison **(B)**. Few cell bodies in the Corpus cerebelli (CCe) **(C)** are EGFP positive, resembling Purkinje cells due to their neurites extending into the molecular

layer. EGFP positive cells can also be seen in the ventral (Hv) **(A)** and dorsal **(D)** periventricular hypothalamus (Hd), as well as in pallium (P) and subpallium (S), and the thalamic region (TR) **(A)**. Within the TeO, EGFP expressing cell bodies reside in the periventricular grey zone (PGZ), within the neuropil layer (arrows) and close to the optic tectum surface (superficial interneurons, arrowheads) **(E)**. Green, EGFP; magenta, BODIPY; blue, DAPI. Scale bars correspond to 500 µm **(A)**, 100 µm **(B2, C2, D2, E2)**, 50 µm **(E4)** and 25 µm **(B6)**. CCe, Corpus cerebelli; GL, granule cell layer of CCe; Hd, dorsal hypothalamus; Hv, ventral hypothalamus; LOT, lateral olfactory tract; ML, molecular layer of CCe; OB, olfactory bulb; ON, optic nerve; P, pallium; PCL, purkinje cell layer; PGZ, periventricular grey zone; S, subpallium; SFGS, superficial grey/fibrous layer of optic tectum; TeO, optic tectum; TR, thalamic region.

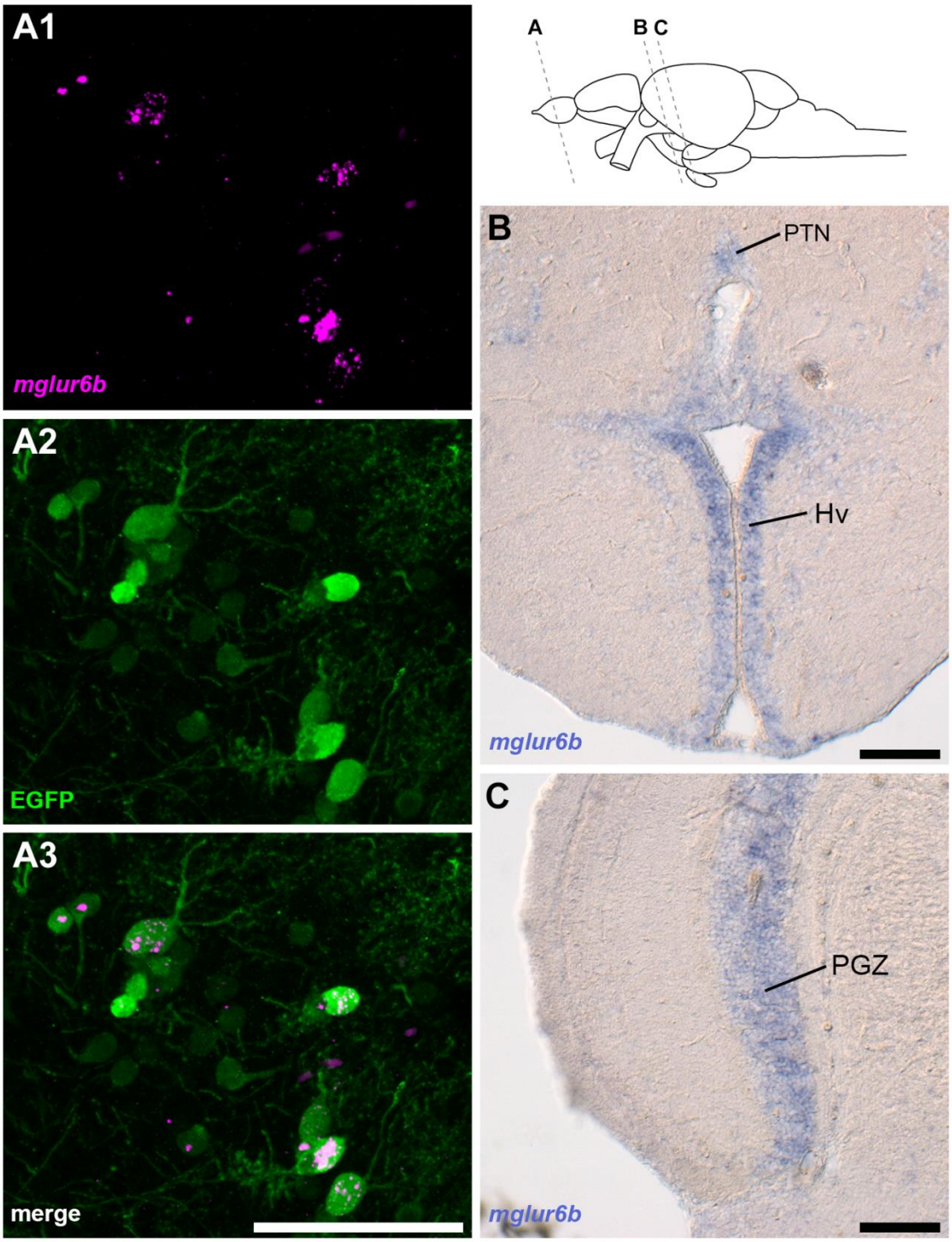

Figure 4. *mglur6b* mRNA expression in the adult zebrafish brain. Confocal images of fluorescent *in situ* hybridization combined with EGFP antibody staining on cross sections of *Tg(mglur6b:EGFP)zh1* olfactory bulb **(A)** and bright field microscopy of chromogenic *in situ* hybridization on cross sections of wild type (wt) Hv **(B)** and TeO **(C)**. In the olfactory bulb **(A)**, both *mglur6b* mRNA (magenta) and EGFP (green) expression is found in ruffed cells and very likely also in mitral cells. In Hv **(B)**, *mglur6b* is detected in the periventricular zone. Also the posterior tuberal nucleus (PTN) expresses mglur6b **(B)**. In TeO **(C)**, *mglur6b* is clearly detected in the PGZ, whereas expression in single cell bodies within the neuropil layer remains questionable. Scale bars correspond to 50 µm. Hv, ventral hypothalamus; PGZ, periventricular grey zone of optic tectum; PTN, posterior tuberal nucleus.

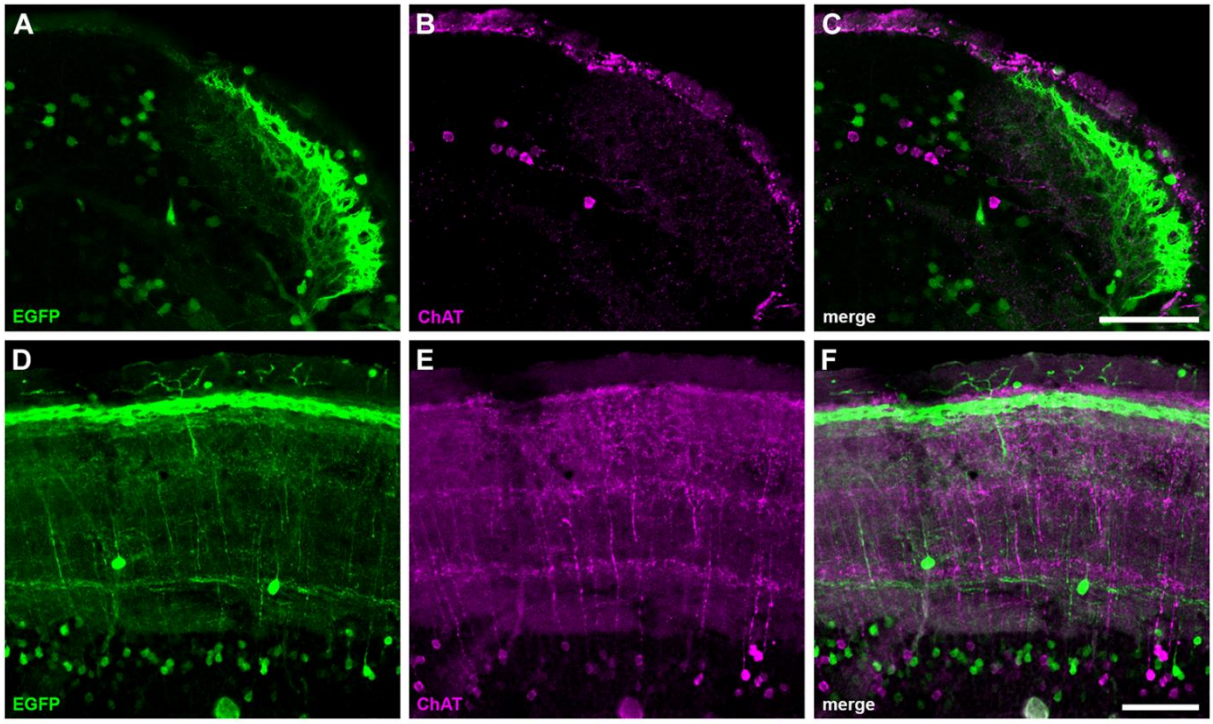

Figure 5. Analysis of EGFP expressing cells in the optic tectum. Double labeling of EGFP (green) and ChAT (magenta) demonstrates non-overlapping *mglur6b:EGFP* expressing and cholinergic subpopulations of TeO neurons in 5-day-old (A-C) and adult (D-F) zebrafish. Scale bars correspond to 50 µm.

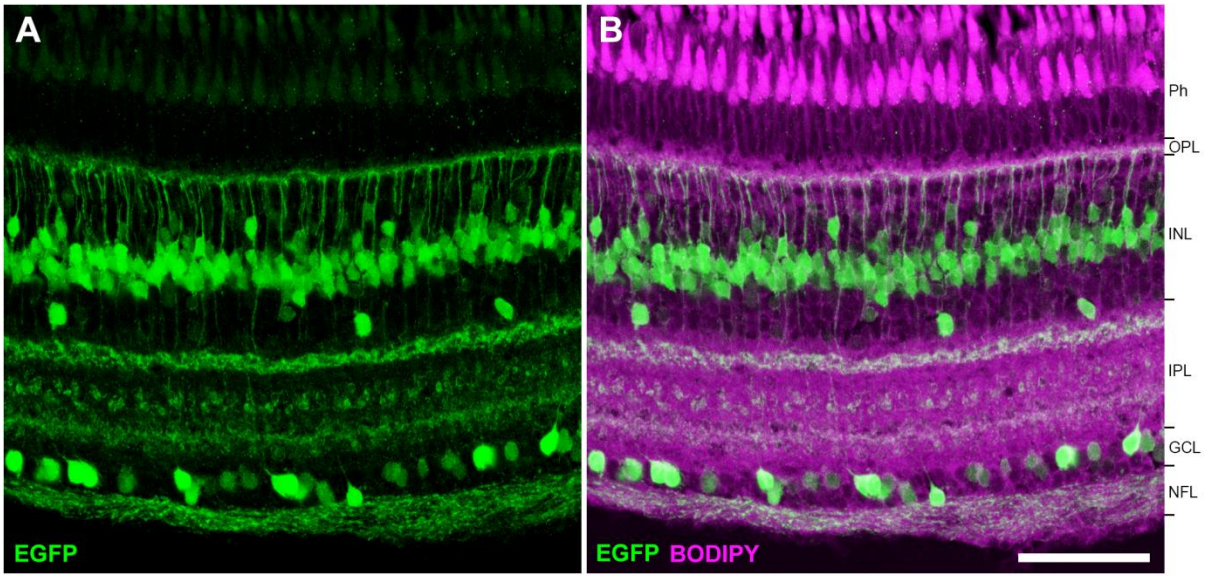

Figure 6. EGFP expression in adult *Tg(mglur6b:EGFP)zh1* zebrafish retina. Confocal image of a retinal section stained with EGFP antibody (green) **(A)** and overlay with BODIPY (magenta) **(B)**. EGFP expressing cells within the INL display morphology and location of bipolar and amacrine or displaced ganglion cells. In the GCL, retinal ganglion cells are EGFP positive, evidenced by labeling of the optic nerve fiber layer. Scale bar corresponds to 50 µm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OPL, outer plexiform layer; Ph, photoreceptor layer.

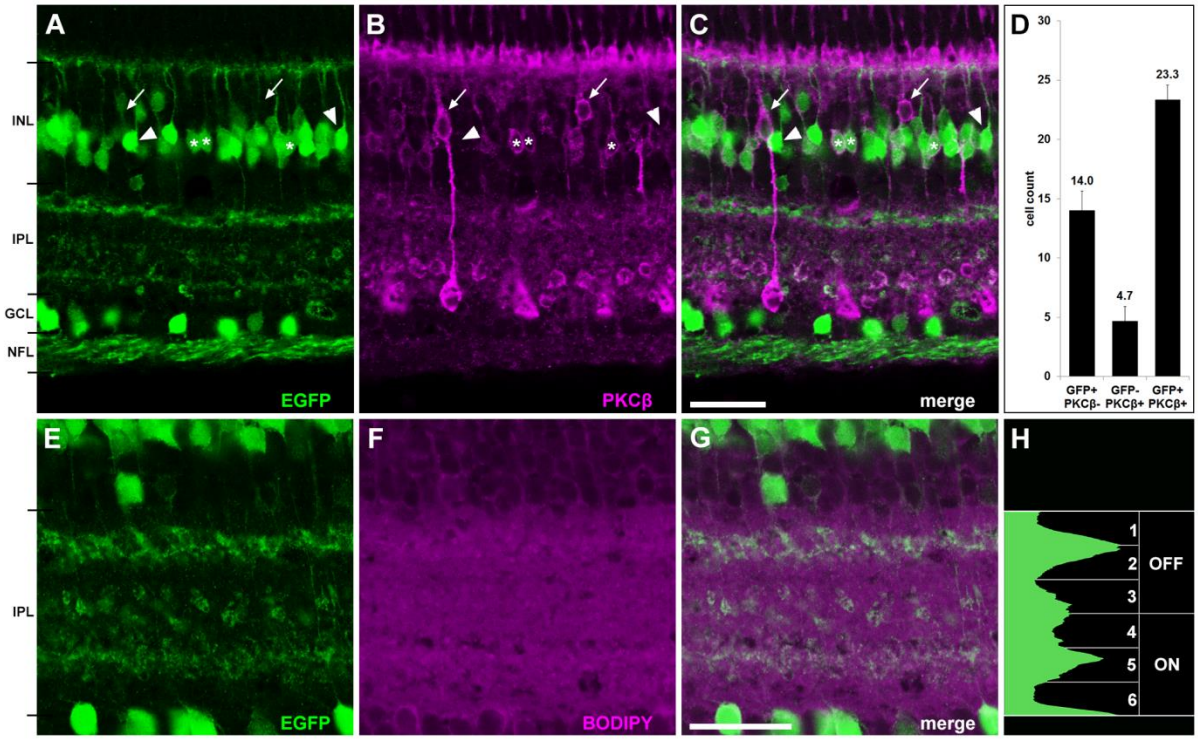

Figure 7. Analysis of EGFP expressing retinal bipolar cells. Double labeling of EGFP and ON bipolar cell marker PKCβ **(A-D)** and labeling of EGFP counterstained with BODIPY with focus on the IPL **(E-H)**. EGFP (green) and PKCβ (magenta) positive cells are substantially overlapping. EGFP positive / PKCβ negative cells and EGFP negative / PKCβ positive cells are also evident **(A-C)**. Note the absence of EGFP in s6L bipolar cells strongly labeled by PKCβ. The experiment was quantified by counting labeled cells in retinal stretches of 150 µm in retinal sections from 3 adult *Tg(mglur6b:EGFP)zh1* zebrafish **(D)**. The entire thickness of the IPL was visualized by staining with BODIPY (magenta) **(F,G)**, and subdivided into 6 sublayers of equal thickness according to Connaughton *et al.* (2004) **(H)**. Fluorescence intensity was quantified as mean intensity value across the inner plexiform layer using ImageJ **(H)**. Axon terminals of EGFP positive bipolar cells (green) with typical bouton-like morphology can be identified in sublayers 3, 4 and 5 of IPL **(E-G)**. EGFP signal at the border between sublayers 1 and 2 is more diffuse and probably represents neurites from other labeled cell types (ganglion cells, amacrine cells), although small bipolar cell axon terminals may also be contained **(E-G)**. Scale bars correspond to 25 µm. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer.

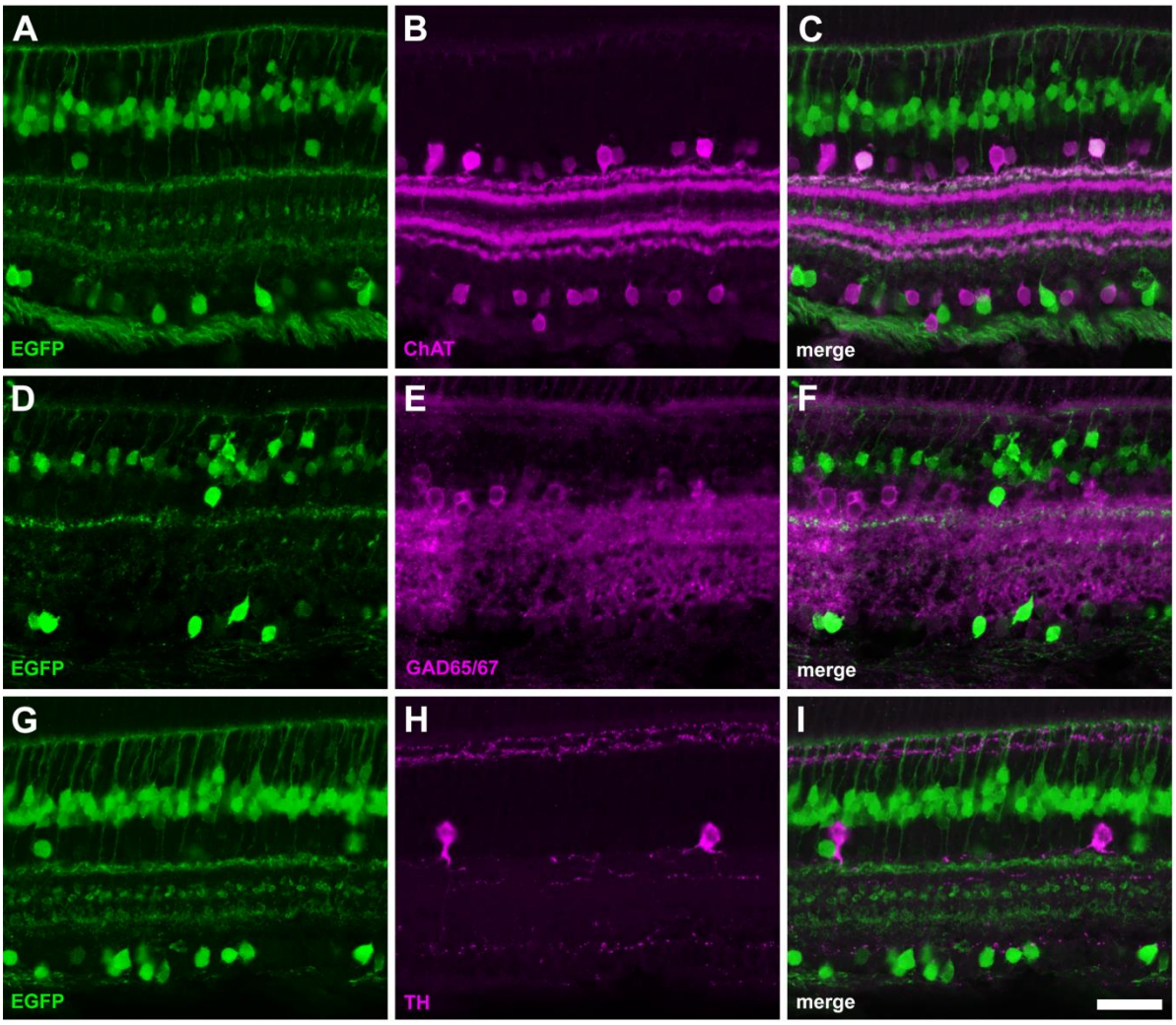

Figure 8. Analysis of EGFP expressing amacrine cells. Double labelings of EGFP with cholinergic cell marker acetylcholinesterase (A-C), GABAergic cell marker GAD65/67 (D-F) and dopaminergic cell marker tyrosine hydroxylase (G-I). All EGFP positive cells (green) with morphology and location of amacrine cells overlap with acetylcholinesterase (magenta) immunoreactivity. Acetylcholinesterase and EGFP staining do not overlap in the ganglion cell layer **(A-C)**. There is no overlap between EGFP (green) and GAD65/67 (magenta) positive cell bodies **(D-F)** or tyrosine hydroxylase (magenta) positive cell bodies **(G-I)**. Scale bar corresponds to 25 µm.

Table 1. Table of primary antibodies used

Antibody	Immunogen structure	Manufacturer, catalog number, RRID, species, monoclonal or polyclonal	Concentration
GFP (chicken)	GFP isolated directly from *Aequorea victoria*	Life Technologies, A10262, AB_2534023, chicken (IgY), polyclonal	1/500
GFP (rabbit)	*E. coli* - expressed full length GFP	Torrey Pines Bioloabs, TP401, AB_10890443, rabbit, polyclonal	1/500
PKCβ1 (C-16)	peptide mapping to C-terminus of human PKC βI	Santa Cruz Biotechnology, sc-209, AB_2168968, rabbit, polyclonal	1/500
ChAT	human placental Choline Acetyltransferase	Millipore, AB144P, AB_2079751, goat, polyclonal	1/100
GAD65/67	peptide near C terminus of human GAD65+67 (aa 572-585)	Abcam, Ab11070, AB_297722, rabbit, polyclonal	1/200
TH	tyrosine hydroxylase purified from rat PC12 cells	Immunostar, 22941, AB_10731005, mouse, monoclonal	1/250